ANTI-IDIOTYPIC ANTIBODIES OF FIBROBLAST GROWTH FACTORS AND THEIR USE AS MEDICAMENTS

The invention relates to anti-idiotypic antibodies of fibroblast growth factors and their use as medicaments.

Fibroblast growth factors (FGFs) are now recognized as the main agents for cellular homeostasis. The role of FGFs has been demonstrated in angiogenesis, tumorigenesis and neuronal degeneration.

Decrease of FGFs causes apoptosis (programmed cell death), and their overabundance causes cells to multiply.

The fibroblast growth factors are a family of peptides of 16-30 kDa that bind to heparin. Examples of members of the FGF family are: acidic fibroblast growth factor aFGF/FGF-1 (Jaye et al., Science 233: 541, 1986), basic fibroblast growth factor bFGF/FGF-2 (Abraham et al., Science 233: 545, 1986), int-2/FGF-3 (Smith et al., EMBO J. 7: 1013, 1988), FGF-4 (Delli-Bovi et al., Cell 50: 729, 1987), FGF-5 (Zhan et al., Mol. Cell Biol. 8: 3487, 1988), FGF-6 (Marics et al., Oncogene 4: 335, 1989); FGF-7 (Finch et al., Science 245: 752, 1989), FGF-8 (Tanaka et al., Proc. Natl. Acad. Sci. USA 89: 8928, 1992) and FGF-9 (Miyamoto et al., Mol. Cell Biol. 13: 4251, 1993).

Patent application WO 90/05184 (CHIRON) describes compositions containing basic and acidic human FGFs, which are acetylated in the amino-terminal position. Applications WO 96/35708 and WO 96/35716 (HOPKINS UNIVERSITY OF MEDICINE) describe homologues of fibroblast growth factors 1 and 2, respectively.

To date, at least 18 genes coding for peptides in this family have been identified. Four different genes code for transmembrane tyrosine kinases identified as FGF receptors, called FGF-R1 to 4. Each of these genes can generate several isoforms by optional splicing of the premessenger RNA. The structure of these genes is conserved, i.e. their extracellular domain is comprised of 2 or 3 domains of the immunoglobulin type and one intracellular domain endowed with tyrosine kinase activity. Thus, the isoform of FGF-R1 that has the 3 domains of the immunoglobulin type (FGF-R1/3 loops) binds FGF2 but not FGF1, whereas the isoform FGF-R1/2 loops binds FGF1 and FGF2 with equal affinity. Furthermore, use of the IIIb exon endows FGF-R2 (FGF-R2b) with the ability to bind FGF1 without binding FGF2, whereas the use of the IIIc exon endows it with the ability to bind FGF1 and FGF2 (Revue Bikfalvi A. Klein S., Pintucci G., Rifkin DM., Endocrine reviews, 1997, 18, 26-45).

A growth factor can produce many different effects, for example proliferation or survival, depending on which of its receptors it binds to. Immunoneutralization can therefore have beneficial effects by inhibiting proliferation, but also undesirable effects by decreasing survival.

Analysis of the functions *in vivo* resulting from the activation of any one of the heparin-binding growth factor receptors, such as the FGFs, comes up against two major stumbling blocks.

On the one hand, the combination of interactions between ligands (such as the FGFs) and receptors is extremely complex, since a growth factor can bind to several receptors and, similarly, several growth factors can bind to the same receptor. Thus, the products of the 18 genes coding for the FGFs have the products of the 4 genes coding for the receptors of the FGFs.

On the other hand, their strong affinity for the glycosamineglycans means that the growth factors such as FGF are sequestered in the extracellular matrices and are only found in the immediate vicinity of their place of synthesis, making it difficult to identify their role *in vivo*.

So far, there are no other ligands apart from the FGFs for each of the isoforms of the FGF receptors. In view of their sequestration in the extracellular matrices, the use of FGFs does not make it possible to identify the function of each of these isoforms *in vivo*, and hence their true role in physiopathology.

One of the aims of the invention is to propose the use of anti-idiotypic antibodies of growth factors with affinity for heparin, permitting the specific targeting of one or other of their receptors, a new type of circulating agonists.

One of the other aspects of the invention is to supply antagonists of growth factor receptors having appropriate specificity and a long half-life.

One of the other aims of the invention is to supply internal images of the domains of binding of the FGFs to their receptors, which because of their immunoglobulin structure are circulating.

More particularly, one of the aims of the invention is to supply internal images of binding domains of FGF1 to FGF-R2b, and of FGF2 to FGF-R1, which because of their immunoglobulin structure are circulating.

One of the other aims of the invention is to propose the use of anti-idiotypic antibodies for stimulating or inhibiting the activity of the receptors of FGF1 and/or of

the receptors of FGF2, or to propose vectors of medicaments of interest through the medium of the receptors of FGF1 and/or of FGF2.

The present invention relates to the use of anti-idiotypic antibodies of fibroblast growth factor 1 and/or anti-idiotypic antibodies of fibroblast growth factor 2, for the preparation of a medicament intended for the treatment of diseases in which endothelial cells are involved in a process of angiogenesis, either for inhibiting angiogenesis, or for promoting angiogenesis, without affecting the quiescent endothelial cells, or for the preparation of a diagnostic product of diseases in which endothelial cells are involved in a process of angiogenesis.

The expression "endothelial cells involved in a process of angiogenesis" signifies endothelial cells migrating across the basal lamina and multiplying.

To determine whether cells are involved in a process of angiogenesis, it is possible to employ immunolabelling using antibodies directed against integrin β3 (Brooks et al., Cell, 1994, 79: 1157-1164) or VEGF-R2 (Ortega N. et al., American Journal of Pathology, Vol. 151, 1215-1224, 1997).

The term "quiescent endothelial cells" signifies endothelial cells of the normal, non-angiogenic, adult vessels.

The invention relates to the use of anti-idiotypic antibodies of fibroblast growth factor 1 and/or anti-idiotypic antibodies of fibroblast growth factor 2, for the preparation of a medicament intended for the treatment of diseases involving angiogenic endothelial cells, by respective selective stimulation of the FGF-R2b and FGF-R1 receptor.

The term "angiogenic endothelial cells" denotes cells involved in a process of angiogenesis.

According to an advantageous embodiment, the invention relates to the use of anti-idiotypic antibodies of fibroblast growth factor 1 and/or anti-idiotypic antibodies of fibroblast growth factor 2, for the preparation of a medicament intended for the treatment of diseases in which endothelial cells are involved in a process of angiogenesis, for inhibiting angiogenesis, without affecting the quiescent endothelial cells, the anti-idiotypic antibody being coupled to a toxin whose function is to block the translation of the proteins, the said toxin being chosen in particular from saporin, ricin or alternatively a radioactive element such as iodine 125 or 131, or the said anti-idiotypic antibody is in the form of an Fab fragment.

According to another advantageous embodiment, the invention relates to the use of anti-idiotypic antibodies of fibroblast growth factor 1 and/or anti-idiotypic antibodies

of fibroblast growth factor 2, for the preparation of a medicament intended for the treatment of diseases in which endothelial cells are involved in a process of angiogenesis, in order to promote angiogenesis, without affecting the quiescent endothelial cells.

Thus, we may mention in particular, according to the invention, the use of anti-idiotypic antibodies of fibroblast growth factor 1 or anti-idiotypic antibodies of fibroblast growth factor 2 for the preparation of a medicament intended to:

– promote physiological angiogenesis for increasing the rate of formation of blood vessels in the course of healing, of maturation of the corpus luteum of the ovary, and/or

– promote angiogenesis in the course of obstructive diseases of vessels for the reperfusion of ischaemic regions during vascular thrombosis especially in lower limb arteritis and myocardial infarction, and/or

– selectively stimulate the activity of the receptors of FGF1 and/or of FGF2 in diseases in which the said receptors are functionally deficient, and/or

– selectively inhibit the activity of the receptors of FGF1 and/or of FGF2 by means of Fab fragments or blocking anti-idiotypic antibodies, and/or

– delay or stop the process of degeneration of the photoreceptors of the neuroretina observed in genetic pigmentary retinitis, either genetic or acquired during overdoses of medicaments inhibiting cyclic-GMP-dependent phosphodiesterase, and/or

– stimulate phagocytosis of the external segments of the rods by the pigmented epithelial cells of the retina as treatment for certain pigmentary retinopathies and of the dry forms of age-related macular degeneration.

In addition, we may mention, according to the invention, the use of anti-idiotypic antibodies of fibroblast growth factor 1 and/or anti-idiotypic antibodies of fibroblast growth factor 2, combined with a toxin or of Fab fragment of anti-idiotypic antibody, for the preparation of a medicament intended for the treatment of diseases requiring the inhibition of angiogenesis, such as cancer, diabetic retinopathies and rejection of cornea transplants.

According to an advantageous embodiment, the invention relates to an anti-idiotypic antibody, especially monoclonal, and especially humanized, of fibroblast growth factor 1, characterized in that it is respectively a ligand of the human FGF-R2b receptor.

More particularly, the invention relates to an anti-idiotypic antibody, especially monoclonal, and especially humanized, of fibroblast growth factor 1, characterized in that it has the following properties:

- it is specific with respect to the FGF-R2b receptor,
- it is circulating,
- it has a half-life of about 23 days, especially of about 21 days, and in particular of 22.5 days,
- it induces the phosphorylation of a protein of 140 kDa on a tyrosine,
- it induces the proliferation of vascular endothelial cells,
- it stimulates angiogenesis,
- it does not cause arterial hypotension.

According to another advantageous embodiment, the invention relates to an anti-idiotypic antibody, especially monoclonal, and especially humanized, of fibroblast growth factor 2, characterized in that it is respectively a ligand of the human FGF-R1 receptor.

More particularly, the invention relates to an anti-idiotypic antibody of fibroblast growth factor 2, especially monoclonal, and especially humanized, characterized in that it has the following properties:

- it is specific with respect to the FGF-R1 receptor,
- it is circulating,
- it has a half-life of about 23 days, especially about 21 days, and in particular 22.5 days,
- it induces the phosphorylation of a protein of 140 kDa on a tyrosine,
- it induces the proliferation of vascular endothelial cells,
- it stimulates angiogenesis,
- it does not cause arterial hypotension.

The anti-idiotypic antibodies of FGF1 of the invention recognize human FGF-R2b receptor, but do not recognize the FGF-R1 receptor.

The anti-idiotypic antibodies of FGF2 of the invention recognize human FGF-R1 receptor but do not recognize the FGF-R2b receptor.

The expression "anti-idiotypic antibody of fibroblast growth factor 1, characterized in that it is specific with respect to the FGF-R2b receptor", signifies that it activates the functions of FGF-R2b.

Similarly, the expression "anti-idiotypic antibody of fibroblast growth factor 2, characterized in that it is specific with respect to the FGF-R1 receptor", signifies that it activates the functions of FGF-R1.

The specificity of the anti-idiotypic antibodies of FGF1 relative to FGF-R2b can be determined by the test of competition with radioiodinated FGF1 relative to its binding to CHO cells transfected with eukaryotic expression vectors containing the sequence of the FGF-R2b receptor.

Similarly, the specificity of the anti-idiotypic antibodies of FGF2 relative to FGF-R1 can be determined by the test of competition with radioiodinated FGF2 relative to its binding to CHO cells transfected with expression vectors containing the sequence of the FGF-R1 receptor.

The term "circulating anti-idiotypic antibody" signifies freely carried in the circulating blood and not captured by the vessel walls.

In contrast to the FGF1 and FGF2 anti-idiotypic antibodies of the invention, the FGF1 and FGF2 growth factors are not circulating.

The advantage of the anti-idiotypic antibodies of the invention being specific and circulating is the targeting of angiogenic endothelial cells with medicaments that do not affect the quiescent endothelial cells.

Regarding the half-life of the anti-idiotypic antibodies, it varies from one species to another.

The half-life of the FGF1 or FGF2 anti-idiotypic antibodies of the invention can be measured by the following test: intravenous injection of the radioiodinated ligand (FGF1 or FGF2), then taking of blood samples at various time intervals and counting the radioactivity. The half-life corresponds to the time required for 50% of the initial radioactivity to disappear from the circulating blood.

The half-life of FGF1 is less than 2 minutes; the half-life of FGF2 is less than 2 minutes. As a guide, the half-life of IgG is of the order of 23 days.

The protein of 140 kDa on which the FGF1 anti-idiotypic antibodies of the invention induce the phosphorylation of a tyrosine is FGF-R2b.

The protein of 140 kDa on which the FGF2 anti-idiotypic antibodies of the invention induce the phosphorylation of a tyrosine is FGF-R1.

This aspect signifies that activation of FGF-R2b by the FGF1 anti-idiotypic antibodies can trigger functions such as proliferation, migration, or resistance to apoptosis, requiring the phosphorylation of FGF-R2b.

Similarly, activation of FGF-R1 by the FGF2 anti-idiotypic antibodies can trigger functions such as proliferation, migration, or resistance to apoptosis, requiring the phosphorylation of FGF-R1.

This aspect can be measured by the phosphorylation test, for the purpose of verifying that the anti-idiotypic antibodies according to the invention are functional, i.e. that they induce phosphorylation at the level of tyrosine residues of FGF receptors, without which there cannot be any biological functions such as proliferation, dissociation, angiogenesis etc.

A conventional method of measuring the activity of phosphorylation of the FGF receptor, for example FGF-R1, consists, firstly, of incubating VSM cells for 24 hours in serum-free medium, then for 10 minutes in the presence or absence of 100 ng/ml of FGF2 or FGF1, or 500 μg/ml of Ig2Id F1 or Ig2Id F2. The cells are then rinsed with a solution of cold phosphate buffer (PBS) then lysed, and the FGF-R1 is immunoprecipitated by means of an anti-FGF-R1 antibody. The complex is separated by electrophoresis on sodium dodecyl sulphate (SDS) gel, transferred onto a nitrocellulose membrane, and the phosphorylation of FGF-R1 is detected using an anti-phosphotyrosine antibody.

Induction of the proliferation of vascular endothelial cells signifies that they multiply. This can be determined by the test described later (see examples, paragraph 3.1 "Mitogenicity").

Stimulation of angiogenesis signifies that binding of FGF1 anti-idiotypic antibodies to the FGF-R2b receptor followed by phosphorylation of FGF-R2b on a tyrosine and cell proliferation are sufficient to trigger angiogenesis.

Similarly, stimulation of angiogenesis signifies that binding of FGF2 anti-idiotypic antibodies to the FGF-R1 receptor followed by phosphorylation of FGF-R1 on a tyrosine and cell proliferation are sufficient to trigger angiogenesis.

This can be quantified by the test described later (see examples, paragraph 3.3 "Corneal angiogenesis").

The invention also relates to the Fab fragment of the FGF1 and/or FGF2 anti-idiotypic antibodies according to the invention.

The invention also relates to the complex between an anti-idiotypic antibody according to the invention (i.e. an anti-idiotypic antibody of fibroblast growth factor FGF1 and/or of fibroblast growth factor FGF2), and a toxin, in particular selected from

saporin, ricin, or alternatively a radioactive element such as iodine 125 or 131, or strontium.

The invention also relates to a method of preparation of an anti-idiotypic antibody of fibroblast growth factor 1 and/or an anti-idiotypic antibody of fibroblast growth factor 2 according to the invention, characterized in that:

a) purified fibroblast growth factor 1 (FGF1) and/or fibroblast growth factor 2 (FGF2) is injected into an animal, especially a rabbit,

b) blood is taken for recovering the purified immunoglobulins (Ig) containing specific anti-FGF1 antibodies (Ig1 F1) and/or anti-FGF2 antibodies (Ig1 F2), for example by protein A affinity chromatography then if necessary the specific Ig1 F1 and/or Ig1 F2 are purified from the purified Ig, for example by affinity chromatography for FGF1 and/or FGF2,

c) the aforesaid purified Ig's and the aforesaid specific purified Ig1 F1 and/or Ig1 F2 are injected into an animal of the same species as that used for injection of FGF1 and/or FGF2, in particular into the popliteal ganglia of rabbit of the same allotype as that which produced Ig1 F1 and/or Ig2 F2, during injection of FGF1 and/or FGF2,

d) blood is taken to recover the total Ig's, for example by protein A, and then to subject the total Ig's to two immunoadsorptions:

- immunoadsorption on an affinity column prepared with the pre-immune Ig's of the rabbit (Ig PI) used for making the Ig1 F1 and/or Ig1 F2, to eliminate the anti-allotype or isotype antibodies,
- immunoadsorption on an affinity column prepared with the Ig1 F1 and/or Ig1 F2, to purify the anti-idiotypic antibodies (Ig2Id F1 or Ig2Id F2).

Step a) injection of FGF1 or of FGF2 into an animal, especially a rabbit, takes place under the skin.

The expression "specific anti-FGF1 antibodies (Ig1 F1)" signifies that the antibodies directed against the FGF1 (anti-FGF1 antibodies) do not recognize the FGF2: in fact, less than 5% of cross reaction with FGF2 is observed, which means that at least 20 times more FGF2 than FGF1 is required for neutralizing the same quantity of anti-FGF1 antibody. As with the specific anti-FGF1 antibodies, the specific anti-FGF2 antibodies (Ig1 F2) do not recognize FGF1: in fact, less than 5% of cross reaction with FGF1 is observed.

The invention also relates to an anti-idiotypic antibody of fibroblast growth factor 1 and/or of fibroblast growth factor 2, which can be obtained by the following method:

a) purified fibroblast growth factor 1 (FGF1) and/or fibroblast growth factor 2 (FGF2) is injected into an animal, especially a rabbit,

b) blood is taken for recovering the purified immunoglobulins (Ig) containing specific anti-FGF1 antibodies (Ig1 F1) and/or anti-FGF2 antibodies (Ig1 F2), for example by protein A affinity chromatography then, if necessary, the specific Ig1 F1 and/or Ig1 F2 are purified from the purified Ig's, for example by affinity chromatography for FGF1 and/or FGF2,

c) the aforesaid purified Ig's or the aforesaid specific purified Ig1 F1 and/or Ig1 F2 are injected into an animal of the same species as was used during injection of FGF1 and/or FGF2, in particular in the popliteal ganglia of a rabbit of the same allotype as that which produced Ig1 F1 and/or Ig2 F2, during injection of FGF1 and/or FGF2,

d) blood is taken for recovering the total Ig's, for example with protein A, then the total Ig's are submitted to two immunoadsorptions:

- immunoadsorption on an affinity column prepared with the pre-immune Ig's of the rabbit (Ig PI) that was used for making the Ig1 F1 and/or Ig1 F2, to eliminate the anti-allotypic or isotypic antibodies,
- immunoadsorption on an affinity column prepared with Ig1 F1 and/or Ig1 F2, to purify the anti-idiotypic antibodies (Ig2Id F1 or Ig2Id F2).

The invention also relates to a method of preparation of a monoclonal anti-idiotypic antibody of FGF1 and/or a monoclonal anti-idiotypic antibody of FGF2 according to the invention, characterized in that:

a) an animal, especially a mouse, is injected with FGF1 and/or FGF2,

b) splenocytes are recovered from the animal synthesizing Ig1 F1 and/or Ig1 F2,

c) the aforesaid splenocytes are fused with myeloma cells,

d) the hybridomas obtained at the end of the preceding step c) are selected for synthesis of immunoglobulins directed against FGF1 and/or FGF2,

e) an animal, and especially a mouse, of the same allotype as that which produced Ig1 F1 and/or Ig1 F2, is injected with the Ig1 F1 and/or Ig1 F2 thus selected at the end of step d),

f) the splenocytes synthesizing Ig2Id F1 and/or Ig2Id F2 are recovered,

g) the cells from the spleen (splenocytes) are fused with myeloma cells,

h) the hybridomas obtained at the end of the preceding step g) are selected for synthesis of Ig2Id F1 directed against Ig1 F1 and/or Ig2Id F2 directed against Ig1 F2,

i) the said Ig2Id F1 and/or Ig2Id F2 are recovered.

According to an advantageous embodiment of the method of preparation described above, of a monoclonal anti-idiotypic antibody of FGF1 and/or of a monoclonal anti-idiotypic antibody of FGF2, the conditions of step a) comprise, firstly, of injecting a mouse with fibroblast growth factor 1 and/or fibroblast growth factor 2, in a quantity varying from 5 to 50 µg of FGF1 and/or FGF2, 3 times subcutaneously at intervals of 15 days, then a fourth time intraperitoneally or intravenously.

After step a) of injection, the spleen is removed from the mouse for recovery of the splenocytes synthesizing Ig1 F1 and/or Ig1 F2. At the time of removal of the spleen, the total splenocytes are fused with myeloma cells. The resulting hybrid cells multiply and we then separate those secreting the antibody of interest. The unfused splenocytes die in 8 days.

Step i) of recovery of the anti-idiotypic antibodies according to the invention consists more particularly of:

– selecting the FGF1 anti-idiotypic antibodies by their capacity for inhibiting the binding of iodinated FGF1 to the FGF-R2b receptor and not inhibiting the binding of iodinated FGF1 to the FGF-R1 receptor,

– selecting the FGF2 anti-idiotypic antibodies by their capacity for inhibiting the binding of iodinated FGF2 to the FGF-R1 receptor and not inhibiting the binding of iodinated FGF2 to the FGF-R2b receptor.

For preparing the Fab fragments of the FGF1 and/or FGF2 anti-idiotypic antibodies of the invention, the procedure described in the manual with the title "Antibodies, a laboratory manual, 628-629, Harlow and David Lane Publishers, Cold Spring Harbor Laboratory, 1998" can be followed.

The invention also relates to pharmaceutical compositions, characterized in that they contain, as active substance, at least one FGF1 and/or FGF2 anti-idiotypic antibody according to the invention, or at least the Fab fragment according to the invention or at least the complex according to the invention, together with a pharmaceutically acceptable vehicle.

The abbreviations used above, and in the examples and diagrams given below, have the following meanings:

PBS: phosphate buffer saline

Ig: immunoglobulins

IgG: immunoglobulin G

Ig PI: rabbit immunoglobulins purified by protein A-Sepharose from blood taken before immunization with FGF1 and/or FGF2.

Ig1 F1: immunoglobulins of rabbit 1 purified by protein A-Sepharose from blood taken after immunization with FGF1 (antibodies directed against FGF1).

Ig1 F2: immunoglobulins of rabbit 1 purified by protein A-Sepharose from blood taken after immunization with the FGF2 (antibodies directed against FGF2).

Ig2Id F1: rabbit 2 immunoglobulins purified by protein A-Sepharose from blood taken after immunization with Ig1 F1 (anti-idiotypic antibodies of FGF1).

Ig2Id F2: immunoglobulins of rabbit 2 purified by protein A-Sepharose from blood taken after immunization with Ig1 F2 (anti-idiotypic antibodies of FGF2).

FGF-Rs: FGF receptors, for example FGF-R1 and FGF-R2b.

According to an advantageous embodiment, the invention relates to the use of FGF1 anti-idiotypic antibodies for:

- stimulating the activity of the receptors of FGF1,
- inhibiting the activity of the receptors of FGF1 with blocking antibodies or Fab fragments,
- coupling Ig2Id F1 to medicaments or genes of interest so as to vectorize them on cells expressing the receptors of FGF1 and stimulate the activity of these receptors,
- coupling Ig2Id F1 to medicaments, toxins or genes of interest so as to vectorize them on cells expressing the receptors of FGF1 and destroy them,
- coupling Ig2Id F1 to radioactive tracers so as to vectorize them on cells expressing the receptors of FGF1 and visualize them in any medical imaging system.

According to another advantageous embodiment, the invention relates to the use of FGF2 anti-idiotypic antibodies for:

- stimulating the activity of the receptors of FGF2,
- inhibiting the activity of the receptors of FGF2 by blocking antibodies or Fab fragments,
- coupling Ig2Id F2 to medicaments or genes of interest so as to vectorize them on cells expressing the receptors of FGF2 and stimulate the activity of these receptors,
- coupling Ig2Id F2 to medicaments, toxins or genes of interest so as to vectorize them on cells expressing the receptors of FGF2 and destroy them,
- coupling Ig2Id F2 to radioactive tracers so as to vectorize them on cells expressing the receptors of FGF2 and visualize them in any medical imaging system.

DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B show the specificity of the Ig2Id F1 and Ig2Id F2 anti-idiotypic antibodies for the FGF-Rs receptors.

More particularly, Figure 1A represents the specificity of the Ig2Id F1 and Ig2Id F2 anti-idiotypic antibodies for the receptor FGF-R1/2 loops, and Figure 1B represents the specificity of the Ig2Id F1 and Ig2Id F2 anti-idiotypic antibodies for the receptor FGF-R1/3 loops.

The abscissa in Figures 1A and 1B shows the concentration of modulators, i.e. the concentration of FGF1, FGF2, Ig2Id F1 and Ig2Id F2, expressed in nM.

The ordinate of Figure 1A represents the binding of iodinated FGF2 on FGF-R1/2 loops (expressed in %) and the ordinate of Figure 1B represents the binding of iodinated FGF2 on FGF-R1/3 loops (expressed in %).

CHO pgsA-745 cells transfected with FGF-R1/2 loops or FGF-R1/3 loops are inoculated with the desired concentrations of FGF1, FGF2, Ig2Id F1 and Ig2Id F2, and 2 ng/ml of FGF2 radioiodinated for 3 h at 4°C. The wells are then rinsed, and the cell carpet is lysed with 0.2 M NaOH as described below.

In Figure 1A and in Figure 1B, the curve with the black diamond (♦) corresponds to FGF2, the curve with the black triangle (▲) corresponds to Ig2Id F1, and the curve with the black square (■) corresponds to Ig2Id F2.

Figure 2 represents the proliferation of aorta smooth muscle cells (VSM), and more particularly the proliferative action of the Ig2Id F1 and Ig2Id F2 anti-idiotypic antibodies on the said cells.

The abscissa in Figure 2 represents the concentration of modulators, i.e. the concentration of FGF1, FGF2, Ig2Id F1 and Ig2Id F2; the concentration of FGF1, FGF2, Ig2Id F1 and Ig2Id F2 is expressed in nM.

The ordinate in Figure 2 represents the number of VSM cells per well ($\times 10^{-3}$).

The curve with the black diamond (♦) corresponds to FGF2, the curve with the black square (■) corresponds to Ig2Id F1, and the curve with the black triangle (▲) corresponds to Ig2Id F2.

The VSM cells are sown at low density (5000 cells/well) in 24-well plates. Variable doses of FGF2, Ig2Id F1 or Ig2Id F2 are added after adhesion of the cells to

the culture plastic. Each condition is studied in two different wells. The cells are trypsinized and counted on the fourth day.

Figure 3 represents inhibition of the mitogenic action of Ig2Id's (FGF1 and/or FGF2 anti-idiotypic antibodies) by the Ig1 F1 and Ig1 F2 antibodies on the VSM cells.

The grey histograms (see those on the left) correspond to absence of antibodies, the shaded histograms (see those in the middle) correspond to the presence of anti-FGF2 antibodies (Ig1 F2), and the black histograms with the white spots (see those on the right) correspond to the presence of anti-FGF1 antibody (Ig1 F1).

The abscissa shows, respectively from left to right: putting in contact of the control or of the Ig1 F1's or of the Ig1 F2's respectively with the control, with FGF2, with Ig2Id F2, with FGF1, with Ig2Id F1.

The ordinate in Figure 3 represents the number of VSM cells per well (x 10^{-3}).

The VSM cells are sown at low density (5000 cells/well) in 24-well plates. 5 ng/ml of FGF1 or FGF2, or 20 µg/ml of Ig2Id F1 or Ig2Id F2, is added after adhesion of the cells to the culture plastic in the presence or absence of 50 µg/ml of Ig1 F1 or Ig1 F2. Each condition is studied in two different wells. The cells are trypsinized and counted on the fourth day.

Figures 4A and 4B show, respectively, the proliferation of cells NBT-II and NBT-II/R1, and more especially, the proliferative action of anti-idiotypic antibodies Ig2Id F1 and Ig2Id F2 on the said cells.

The abscissa in Figures 4A and 4B represents the concentration of modulators, i.e. the concentration of FGF1, FGF2, Ig2Id F1 and Ig2Id F2, expressed in ng/ml.

The ordinate in Figures 4A and 4B represents the number of cells per well ($\times 10^{-3}$).

The curve with the black diamond (♦) in Figures 4A and 4B corresponds to FGF1, and the curve with the black square (■) in Figures 4A and 4B corresponds to Ig2Id F1.

The curve with the black triangle (▲) in Figure 4B corresponds to FGF2, and the curve with the cross in Figure 4B corresponds to Ig2Id F2.

The NBT-II or NBT-II/R1 cells are sown at 5000 cells/well in 24-well plates. Variable doses of FGF1, FGF2, Ig2Id F1 or Ig2Id F2 are added after adhesion of the

cells to the culture plastic. Each condition is studied in two different wells. The cells are trypsinized and counted on the fourth day.

Figure 5 represents the dissociation of the NBT-II and NBT-II/R1 cells.

The NBT-II or NBT-II/FGF-R1 cells are sown at low density in 12-well plates (5000 cells per well). On the next day the modulators, i.e. FGF1, FGF2, Ig2Id F1 and Ig2Id F2 are added to the medium and, on the fourth day, the cells are observed and photographed with the NIKON diaphot inverted phase-contrast microscope.

Figure 6 represents corneal angiogenesis.

The grey rectangles (on the left) correspond to the angiogenesis score on day D7, and the shaded rectangles (on the right) correspond to the angiogenesis score on day D14.

The abscissa represents, respectively from left to right, the control, FGF2, Ig2Id F1, Ig2Id F2.

The ordinate represents the angiogenesis score.

A 3 mm long incision, through half the thickness of the cornea, is made in the corneal dome, under an operating microscope. The corneal stroma is cleaved, in diametrically opposite directions, as far as 2 mm from the edge. The implants, previously rehydrated with 2 µl of PBS containing 30 µg of Ig2Id F1 and Ig2Id F2.

After 14 days, the neovascularization is quantified. Each modulator was studied in at least 4 eyeballs of 4 separate rabbits (8 lenses). The differences in angiogenesis score between each condition and the control lenses are evaluated by the Student t-test.

Figures 7A and 7B show respectively the tumoral growth of the NBT-II and NBT-II/R1 cells in the nude mouse.

The abscissa in Figures 7A and 7B represents the number of days after implantation in the nude mouse, and the ordinate represents the tumour volume (in mm^3).

The curve with the black square (■) corresponds to Ig2Id F2, the curve with the black triangle (▲) corresponds to Ig2Id F1, and the curve with the black diamond (♦) corresponds to the control.

3.5 million NBT-II or NBT-II/R1 cells are injected under the skin of the right flank of 2 groups of 30 female nude mice aged 6 weeks. Four days later, each of the

previous 2 groups is divided into 3 groups of 10 mice taken at random, and the dimensions of any tumours are measured using a slide caliper. 500 µg of Ig2Id F1 or of Ig2Id F2 diluted to a final volume of 100 µl of phosphate buffer + gelatin (2 mg/ml) is injected intravenously in the veins of the tail. This injection is repeated every 3 days for 50 days. The measurements are effected at the same intervals by an operator who does not know the treatment received by each group of mice in each of the 6 groups.

METHODS OF INVESTIGATION

EXAMPLE:

1. Production of anti-idiotypic antibodies of FGF1 and FGF2

1-1 Production of pre-immune antibodies (Ig PI).

Before each immunization, blood is taken, the serum is fractionated immediately after collection and 15 ml of serum is chromatographed on a column of protein A (0.9 × 18 cm). The column is washed with PBS and the immunoglobulins are eluted with 0.2 M glycine buffered to pH 2.5, neutralized immediately by adding 1/5 of the volume of 1 M K_2HPO_4, then dialysed against PBS. The immunoglobulins (Ig PI) are stored at -80°C until they are used.

1-2 Production of anti-FGF1 (Ig F1) and anti-FGF2 (Ig F2) antibodies

100 µg of FGF1 is emulsified in 0.25 ml of Freund complete adjuvant then injected into a rabbit, 4 times at intervals of 15 days. The blood taken between 3 and 7 months after the first injection is fractionated and the Ig's are purified by protein A affinity chromatography (Ig1 F1). These antibodies neutralize the activity of FGF1 without inhibiting that of FGF2.

Following the same protocol, anti-FGF2 neutralizing antibodies (Ig1 F2), which do not neutralize the activity of FGF1, were produced.

1-3 Production of anti-idiotypic antibodies of FGF1 (Ig2Id F1) and of FGF2 (Ig2Id F2).

The animals are premedicated, and 1 ml of a solution of Evans Blue is injected into the sole of the hind feet; 15 minutes later the rabbits are anaesthetized. The

popliteal hollows are shaved and disinfected with Betadine. A horizontal incision of 2 cm, centred on the popliteal hollow, is made with scissors, then the cellular spaces are slit. From one to three ganglia with diameter of 2 mm are identified by their blue staining and 10 μg of Ig1 F1 mixed volume by volume with the Freund complete adjuvant is injected into them using a Hamilton microsyringe at a final volume of 100 μl and a quantity of primary antibody of 20 μg. The procedure is repeated on the other foot.

From four to five repetitions every three weeks are effected percutaneously using a volume-by-volume emulsion of immunogen (Ig1F1 or Ig1F2) and Freund incomplete adjuvant. 40-ml blood samples are taken every three weeks from the 4th month to the 9th month.

The blood taken between 4 and 7 months after the first injection is purified as described previously. The anti-idiotype Ig's (Ig2Id F1) are then purified by protein A affinity chromatography.

Following the same protocol, Ig1 F2's were injected, and gave rise to the formation of anti-idiotypic antibodies of FGF2 (Ig2 F2).

Sorting and/or purification of the anti-idiotypic antibodies according to the invention is effected as described previously, by submitting the total Ig's to two immunoadsorptions (see step d) described previously in the method of preparation of the anti-idiotypic antibodies).

2. Investigation of the specificity of the anti-idiotypic antibodies according to the invention

CHO pgsA 745 cells, native or transfected with FGF-R1/2 loops or FGF-R1/3 loops sown at 30 000 cells per 2 cm^2 well, are cultivated in DMEM medium (Dulbecco's Modified Eagle Medium) containing 10% of foetal calf serum, 100 IU/ml penicillin and 50 μg/ml streptomycin.

The binding of radioiodinated FGF2 (1.10^5 to 2.10^5 cpm/ng) to the transfected cells is measured at 4°C. The cells are washed twice with the binding buffer (DMEM containing 20 mM Hepes (N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid) and 2 mg/ml of gelatin adjusted to pH 7.4). 2 ng/ml of iodinated FGF2 is added with

variable concentrations of FGF1 or of unlabelled FGF2 or of Ig2Id F1 or Ig2Id F2 to a final volume of 0.5 ml.

Non-specific binding is determined in the presence of an excess (500 ng) of purified FGF2. Total and non-specific binding is determined in duplicate. After two hours, the cells are washed 3 times with cold buffer and lysed with 0.5 ml of 0.2 M NaOH. The iodine 125 contained in the dissolved material is counted in a gamma counter.

3. Biological activities of the anti-idiotypic antibodies according to the invention

3.1 Mitogenicity

Aorta smooth muscle cells (VSM) are sown at low density (5000 cells/well) in 24-well plates. The modulators FGF1, FGF2, Ig2Id F1 and Ig2Id F2 are added after adhesion of the cells to the culture plastic, and after 2 days. Each condition is studied in two different wells. The cells are trypsinized and counted on the fourth day.

Ig2Id F1 and Ig2Id F2 trigger a mitogenic effect on the VSM cells.

3.2 Cell differentiation

The NBT-II or NBT-II/FGF-R1 cells are sown at low density in 12-well plates (5000 cells per well). On the next day, FGF1, FGF2, Ig2Id F1 and Ig2Id F2 are added to the medium, and on the fourth day the cells are observed and photographed in the NIKON Diaphot inverted phase-contrast microscope.

3.3 Corneal angiogenesis

The premedicated rabbits are anaesthetized. The eye is externalized and immobilized with the aid of a latex membrane, which has a 1 cm long slit at its centre. An incision 3 mm long, through half the thickness of the corneal stroma, is made in the corneal dome, under an operating microscope (OPMI microscope, Zeiss). The corneal stroma is cleaved, in diametrically opposite directions, as far as 2 mm from the edge. The implants previously rehydrated with 20 µl of the solution to be tested are inserted (FGF2 200 ng, anti-idiotypic antibodies or control antibodies 40 µg).

The appearance of neovessels, arising from limbic vascularization, is studied in single-blind conditions (without knowing the rabbit's number, corresponding to the

substance studied) after 7 and 14 days under general anaesthesia, and is quantified on a scale with five levels, or angiogenesis score.

Score 0 = absence of neovessels,

Score 1 = neovessels not reaching half the distance between the implant and the edge,

Score 2 = neovessels reaching half the distance,

Score 3 = neovessels exceeding half the distance but not invading the implant,

Score 4 = neovessels reaching and invading the implant.

The rabbits are then sacrificed and the eyeballs are removed and fixed in Bouin liquid, for histological analyses.

Each modulator (FGF1, FGF2, Ig2Id F1 and Ig2Id F2) was studied in at least 4 eyeballs of 4 separate rabbits (8 lenses). The differences in angiogenesis score between each of the conditions tested and the control lenses are evaluated by the Student t-test.

3.4 Tumour growth

Growth in the nude mouse of the bladder carcinoma strain

The NBT-II and NBT-II/FGF-R1 cells are detached from the culture plastic using trypsin-EDTA, homogenized, centrifuged and suspended in culture medium to which 10% of foetal calf serum has been added. 1 ml, corresponding to 3.5 million cells, is injected under the skin of the right flank of each mouse. The viability of the suspension is verified beforehand by vital staining with Trypan Blue; the stain is excluded from more than 99% of the cells. Two groups of 30 female nude mice, aged 6 weeks, were implanted with NBT-II or NBT-II/FGF-R1 cells.

4 days later, each of the above 2 groups was divided into 3 groups of 10 mice, selected at random. The dimensions of any tumours were measured with an electronic sliding caliper, and the modulators (FGF1, FGF2, Ig2Id F1 and Ig2Id F2) in a final volume of 100 µl of phosphate buffer + gelatin (2 mg/ml) were injected intravenously in the veins of the tail (FGF1 and FGF2 anti-idiotypic antibodies: 500 µg; non-immune antibodies: 500 µg/injection).

The injections are repeated twice per week, and the measurements are performed at the same intervals by an operator who does not know the treatment received by each group of mice in each of the 6 groups.

At the end of these three types of studies in the nude mouse, the animals are sacrificed. The tumours and some healthy organs (kidney, liver, bladder) are removed, half being preserved in preserving liquid (FAE: Formol 4%, ethanol 40%, acetic acid 10%, H_2O to 100%) and half being frozen by immersion in liquid nitrogen after protection with isothiopentane.

4. Results

4.1 Specificity of Ig2Id F1 and Ig2Id F2 for the FGF-Rs (Figures 1A and 1B)

The natural ligands FGF1 and FGF2 inhibit the binding of iodinated FGF2 on FGF-R1/2 loops. The internal image Ig2Id F1 of FGF1 inhibits the binding of iodinated FGF2 to the CHO cells transfected by FGF-R1/2 loops whereas the internal image Ig2Id F2 of FGF2 inhibits the binding of iodinated FGF2 to the CHO cells transfected either by FGF-R1/2 loops or by FGF-R1/3 loops.

Taking into account a specific fraction estimated at 1% of the immunoglobulins obtained after purification on protein A, the inhibitions observed are comparable in terms of molarity. The plateau is obtained with 10 ng/ml (0.5 nM) of FGF2 and 10 µg/ml (0.6 nM) of Ig2Id F1 and Ig2Id F2.

On the other hand, neither FGF1 nor Ig2Id F1 inhibit the binding of iodinated FGF2 to FGF-R1/3 loops. The plateau is reached at 3 nM of FGF2 or Ig2Id F2.

Ig2Id F2 is therefore an internal image of FGF2, and Ig2Id F1 is an internal image of FGF1.

4.2 Cellular proliferation (Figures 2, 3, 4A and 4B)

Primary culture VSM (Figure 2): FGF1 and FGF2 induce a dose-dependent proliferation.

Ig2Id F1 and Ig2Id F2 have effects comparable to that of FGF2. Maximum growths are obtained for 1.2 nM.

Proof of the anti-idiotypic nature is supplied by the observation that the mitogenic action of Ig2Id F1 (like FGF1) is inhibited by Ig1 F1 but not by Ig1 F2. On the other hand, Ig2Id F2 is inhibited by Ig1 F2 but not by Ig1 F1 (Figure 3).

Strains NBT-II and NBT-II/FGF-R1 (Figures 4A and 4B): neither FGF2 nor Ig2Id F2 inhibits the growth of the NBT-II cells as these cells do not express the FGF-R1

receptor. Although these cells express the FGF-R2 receptor, Ig2Id F1 does not inhibit proliferation, whereas FGF1 (0.05 nM) does.

In contrast, FGF1, FGF2, Ig2Id F1 and Ig2Id F2 induce a decrease in the number of NBT-II/FGF-R1 cells, corresponding to 30% of the values of the control wells.

4.3 Cell differentiation and effect of dissociation (Figure 5)

Cell differentiation is compared in the sense of acquisition of a mesenchymatous phenotype under the effect of the FGFs.

It is observed that neither FGF2 nor Ig2Id F2 induce differentiation of the NBTII cells, as they do not express the FGF-R1 receptor.

Ig2Id F1 and FGF1 induce the dissociation of the NBT-II cells.

On the other hand, FGF1, FGF2, Ig2Id F1 and Ig2Id F2 induce a dissociation of the NBT-II/FGF-R1 cells.

4.4 Corneal angiogenesis (Figure 6)

In the corneal angiogenesis model, the non-immune control immunoglobulins lead inconsistently (15% of cases) to minor angiogenesis leading to an average score of 0.4 on D15, comparable to that obtained by saturating the lenses with a carrier protein (bovine albumin serum).

10 pm/implant of FGF1 and FGF2 induces significant angiogenesis (scores of 2.35 and 2.05 respectively).

Ig2Id F1 and Ig2Id F2 at a dose of 4 pm/implant lead to angiogenesis (scores of 1.3 and 1.85 respectively on D15) significantly greater than that obtained with the control immunoglobulins ($p < 0.05$).

The kinetics of appearance of neovessels seems different from that obtained with the natural ligand.

On D7, FGF2 reaches the threshold of statistical significance with 80% of the maximum effect, whereas the effects produced by Ig2Id F1 and Ig2Id F2 are at 10% and 41% of the maximum scores.

Ig2Id F1 and Ig2Id F2 induce angiogenesis like the ligands FGF1 and FGF2.

4.5 Tumour growth (Figure 8)

Neither Ig2Id F1 nor Ig2Id F2 affects the growth of xenotransplants of NBT-II cells.

On the other hand, although the growth of NBT-II/FGF-R1 cells is not affected by Ig2Id F1, it is inhibited by Ig2Id F2, which reproduces the inhibitory effect on proliferation observed in vitro after inoculation of FGF2 or Ig2Id F2.